The Thiazoline-Specific Amidohydrolase PurAH is the Gatekeeper of Bottromycin Biosynthesis

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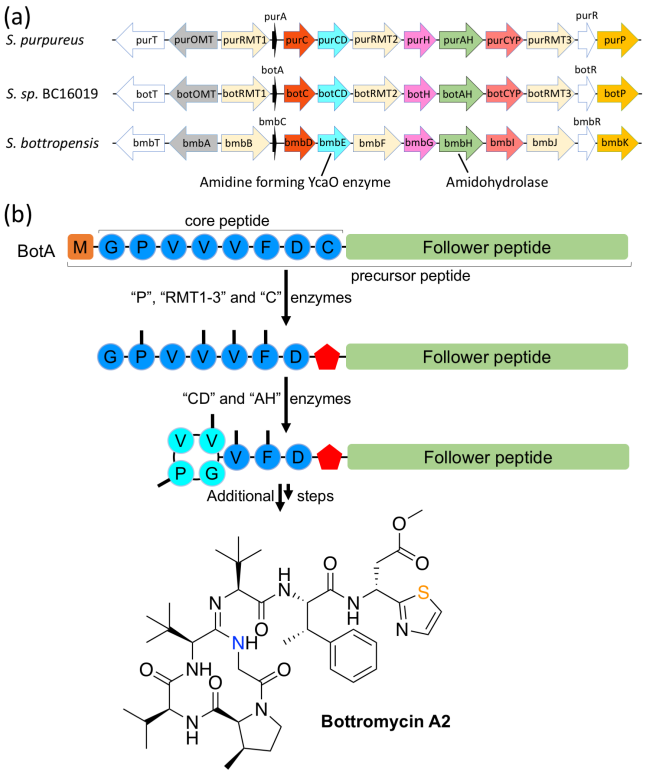
Supporting Information Placeholder

ABSTRACT: The ribosomally synthesized and post-translationally modified peptide (RiPP) bottromycin A2 possesses potent antimicrobial activity. Its biosynthesis involves the enzymatic formation of a macroamidine, a process previously suggested to require the concerted efforts of a YcaO enzyme (PurCD) and an amidohydrolase (PurAH) *in vivo*. *In vitro*, PurCD alone is sufficient to catalyze formation of the macroamidine, but the process is reversible. We set out to probe the role of PurAH in macroamidine formation *in vitro*. We demonstrate that PurAH is highly selective for macroamidine-containing precursor peptides and cleaves C-terminal of a thiazoline, thus removing the follower peptide. After follower cleavage, macroamidine formation is irreversible, indicating PurAH as the gatekeeper of bottromycin biosynthesis. The structure of PurAH suggests residues involved in catalysis, which were probed through mutagenesis.

Bottromycins[1-2] are natural product antibiotics with activity against problematic human pathogens such as Methicillin-resistant Staphylococcus aureus (MRSA)[3-4]. They bind to the A-site of the prokaryotic 50S ribosome, which is a novel antibiotic target[5-7]. Bottromycins belong to the growing family of ribosomally synthesized and post-translationally modified peptides (RiPPs), and their biosynthesis and total synthesis have received increasing attention[8-12]. They are derived from the precursor peptide BotA, which undergoes a series of post-translational enzymatic tailoring steps, the order of which has been proposed based on an untargeted metabolomics approach using mass spectral networking (Scheme 1, we will use *Streptomyces sp.* BC16019 nomenclature)[13]: First, the N-terminal methionine is removed by a leucyl-amino peptidase (BotP), followed by heterocyclization of the BotA cysteine residue to thiazoline by the YcaO enzyme BotC and Cβ-methylation by radical methyl transferases 1-3. Next, a second YcaO enzyme, BotCD, was reported to act together with the metallo-dependent amidohydrolase BotAH in macroamidine formation[13]. Removal of the follower peptide by the α/β-hydrolase BotH, successive oxidative decarboxylation of the thiazoline to a thiazole (BotCYP) and O-methylation of an aspartate (BotOMT) complete bottromycin biosynthesis.

Scheme 1. (a) A gene cluster highly homologous in sequence and organization to those of confirmed bottromycin producers *Streptomyces sp.* BC16019 and *S. bottropensis* was found in *S. purpureus* (top). Intergenic regions not drawn to scale. (b) Bottromycin biosynthesis as proposed by untargeted metabolomics[13]. Methylation by radical methyl transferases (RMT1-3) is shown as black lines, heterocyclized cysteine as a red pentagon and the macroamidine in cyan.



In vitro work has confirmed the assigned functions for BotP[13-14], BotC[8-9] and BotCD[8-9]. It was demonstrated that the BotCD homologs BmbE and PurCD alone are sufficient for macroamidine formation *in vitro*[8-9]. For PurCD, macroamidine formation was reversible: It catalyzes both, macroamidine formation and its re-opening[8].

To probe the role of the amidohydrolase in bottromycin biosynthesis *in vitro*, we attempted to express BotAH (insoluble). The close homolog PurAH (72 % sequence identity to BotAH) from *Streptomyces purpureus* (Scheme 1) could be expressed and purified. We first tested possible substrates and incubated PurAH with precursor peptide BotA, BotA^{P} (N-terminal methionine removed), BotA^{PC} (BotA^{P} with heterocyclized cysteine) and BotA^{PCCD} (macrocyclized BotA^{PC}) (Figure 1a).

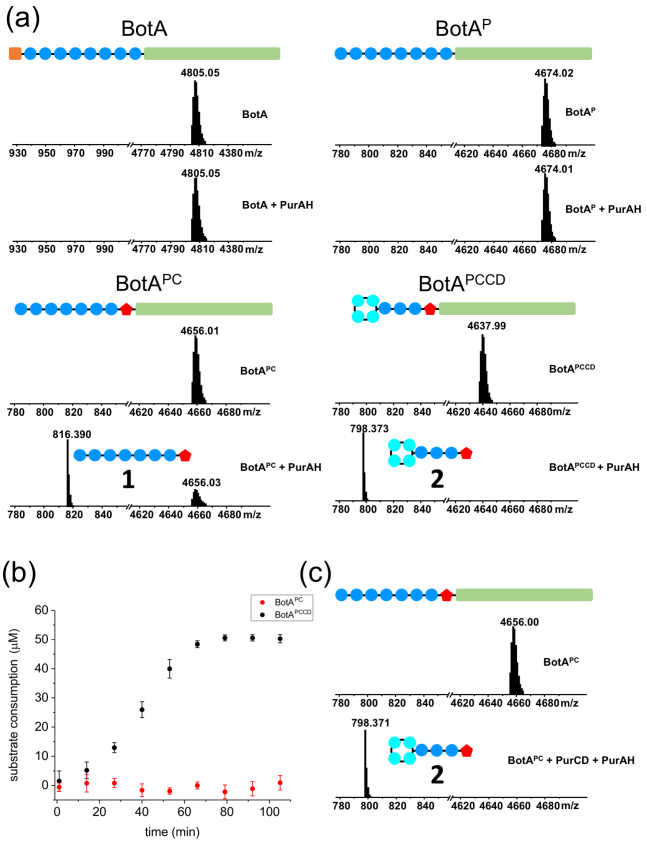


Figure 1. LC-ESI-MS characterization of PurAH reactions. (a) Incubation of PurAH with BotA, BotA^{P}, BotA^{PC}, and BotA^{PCCD} (5 µM enzyme, 50 µM substrate, 37 °C, 16 h). Only BotA^{PC} and BotA^{PCCD} were substrates of PurAH. Colors correspond to scheme 1. (b) Time-course of BotA^{PC} and BotA^{PCCD} (50 µM) cleavage by PurAH (0.2 µM) in the presence of Co^{2+} (100 µM). (c) When PurAH is added to a macrocyclization reaction, the reaction goes to completion and **2** is formed.

All samples were analyzed by liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS). We observed no activity on BotA or BotA^{P}. For the BotA^{PC} reaction, we observed two new masses, which corresponded to the heterocyclized BotA core peptide (**1**, Figure S1A) (m/z calc$_{mono.}$: 816.3840 Da, observed: 816.3814 Da, error -3.2 ppm) and the follower peptide (m/z calc$_{mono.}$: 3857.62 Da, observed: 3857.63 Da, error 2.6 ppm), but the reaction was incomplete. For the BotA^{PCCD} reaction, the substrate was consumed completely and two new masses appeared: One corresponded to the heterocyclized, macroamidine-containing BotA core peptide (**2**, Figure S1b), while the other corresponded to the follower peptide (m/z calc$_{mono.}$: 798.3734 Da, observed: 798.3706 Da, error -3.5 ppm and m/z calc$_{mono.}$: 3857.62 Da, observed: 3858.63 Da, error 2.6 ppm), respectively. The identity of **1** and **2** was confirmed by MS^2 (Figure S2, Tables S1 and S2). These data demonstrate that PurAH is responsible for removing the follower peptide during bottromycin biosynthesis. Since the protein is annotated as a metal-dependent amidohydrolase, we dialyzed purified PurAH extensively with EDTA to remove endogenous metal ions and set up reactions of PurAH with BotA^{PCCD} using different divalent metal ions (Figure S3). No activity was observed for $CdCl_2$ and $NiCl_2$, while addition of $FeCl_2$ or $MgCl_2$ accelerated the background rate slightly. $MnCl_2$ << $ZnCl_2$ < $CoCl_2$ each led to appreciable turnover, with Co^{2+} giving best activity. A time course experiment with 0.2 µM PurAH and 50 µM BotA^{PC} or BotA^{PCCD} revealed that the reaction using BotA^{PCCD} was complete after 80 min, while no product formation was detectable in the same time frame using BotA^{PC} (Figure 1b). PurAH is thus highly selective for the heterocyclized, macrocyclized intermediate BotA^{PCCD} and removes the follower peptide.

With the function of the amidohydrolase established we investigated its involvement in macroamidine formation. The macroamidine forming YcaO enzyme PurCD converts BotA^{PC} into BotA^{PCCD} in an ATP/Mg^{2+}-dependent reaction for which the BotA follower peptide is essential[8-9]. We have shown that this reaction is reversible – purified BotA^{PCCD} incubated with PurCD and ATP/$MgCl_2$ will be converted back to BotA^{PC}, now lacking the macrocycle (Figure S4)[8]. Given the very strong preference of PurAH for BotA^{PCCD} and the dependence of PurCD activity on the presence of the follower peptide that is removed by PurAH, we hypothesized that PurAH may prevent re-opening of the macrocycle. First, we purified **2** and incubated it with PurCD and ATP/$MgCl_2$. Even after extensive incubation times at 37 °C, we did not observe reopening of the macroamidine to yield **1** (Figure S4). BotA^{PCCD} on the other hand converted readily back to BotA^{PC} (Figure S4). When BotA^{PC} is incubated with PurCD under optimized conditions, the turnover to BotA^{PCCD} does not exceed 70 %.[8] However, when PurAH is added to the reaction, we observe complete conversion of BotA^{PC} to **2**, indicating complete conversion of BotA^{PC} (macroamidine formation) and subsequent removal of the follower peptide (Figure 1c). These data rationalize why the amidohydrolase was reported as essential for macroamidine formation *in vivo*[13] – knocking out PurAH likely leads to reopening of the macroamidine and prevented downstream processing. This places PurAH as the gatekeeper of bottromycin biosynthesis, that removes the follower peptide once all enzymes requiring the follower have acted on the precursor peptide.

To understand how PurAH selects for the macrocycle in BotA^{PCCD}, we determined the PurAH crystal structure to 1.73 Å resolution. All data collection and refinement statistics can be found in Table S3. The refined model contained one protomer in the asymmetric unit and includes residues 9 – 74 and 78 – 460

(Figure S5). Two Zn^{2+} ions are coordinated at the active site: Zn1 by His210 and His229, Zn2 by His94, His96 and Asp348. Lys183 has been carboxylated as commonly observed in amidohydrolases[15], with each oxygen coordinating one Zn^{2+} (Figure S5). The distorted trigonal bipyramidal coordination of the two Zn^{2+} is completed by ordered water molecules, one of which bridges the two Zn^{2+} ions. This bridging water may be an activated hydroxyl known to facilitate catalysis in amidohydrolases[16-19]. We generated a sequence similarity network (SSN) for PurAH (Figure S6a) and found it to be part of a small node that exclusively contained PurAH homologs from all known bottromycin biosynthetic gene clusters. Mapping the sequences of this node onto the PurAH structure using ConSurf[20-23], we found residues surrounding the active site to be highly conserved (Figure 2a). In contrast, the closest structural homologs identified by a DALI[24] search show virtually no sequence conservation at the active site (Figure 2b). PurAH contains an extended, wide binding site that may have evolved specifically to accept branched cyclic peptide substrates (Figure S6b).

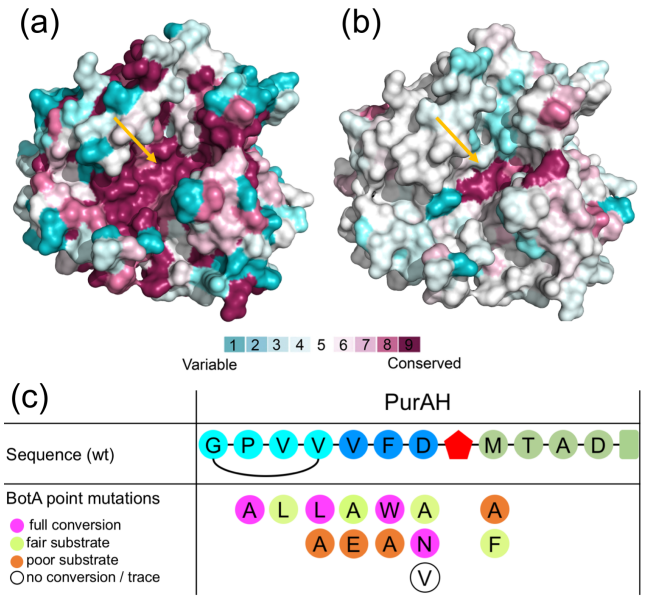
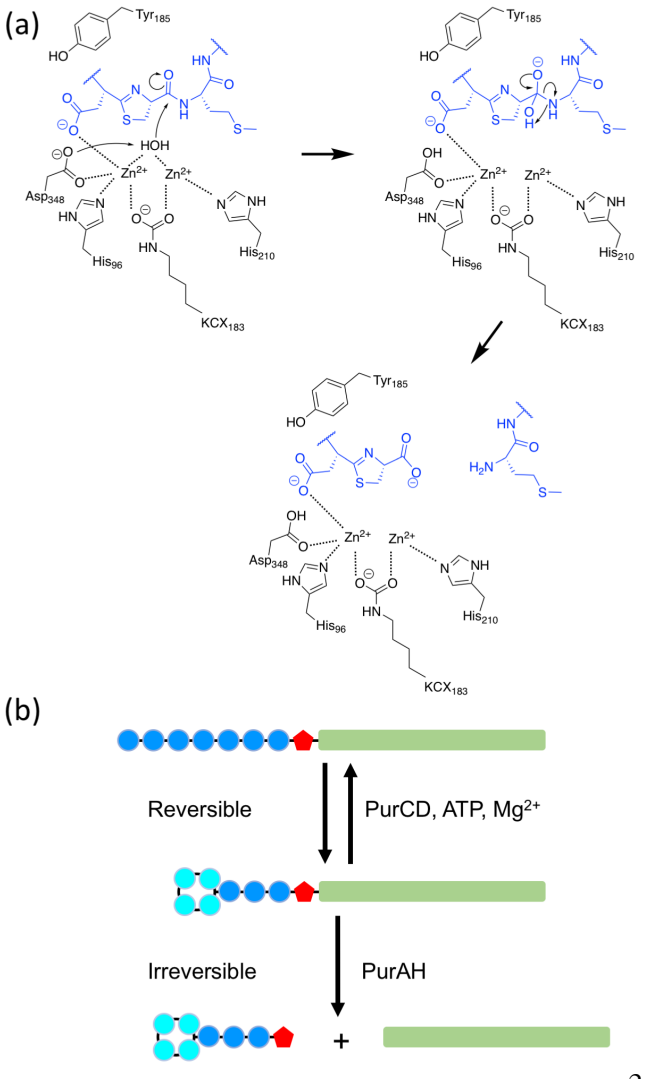


Figure 2. (a) and (b) Consurf maps showing the conservation of residues around the PurAH active site (arrow) for (a) PurAH homologs from other bottromycin biosynthetic gene clusters (PurAH containing node, Figure S6a) and (b) the closest structural homologs identified by a DALI search (Figure S6b). (c) Schematic view of the conversion of 13 BotA mutants designed to test PurAH promiscuity.

To rationalize PurAH's selectivity, we sought to determine the complex crystal structure of PurAH with its substrate, but extensive screening failed in delivering suitable conditions. In most RiPP systems the core peptide sequence is at least to some degree variable, but in all bottromycin biosynthetic gene clusters identified to date the core peptide sequence is fully conserved[25-27] (with the exception of a single-amino acid change found in one cluster[28]). This is at odds with the substrate promiscuity observed for bottromycin biosynthetic enzymes *in vitro* thus far[8-9,14]. To probe the promiscuity of PurAH and the fidelity for macrocyclized intermediate, we generated a series of mutant precursor peptides (Figure 2c). The degree of conversion was estimated by monitoring the consumption of substrate (see SOI for details). Data for the mutations are provided in Figures S7 – S20. BotA mutants Pro2Ala, Val4Leu, Phe6Trp, and Asp7Asn were found to be good substrates of PurAH (Figure 2c). The mutants Val3Leu, Val5Ala, and Asp7Ala were fair and Val4Ala, Val5Glu, and Phe6Ala were poor substrates of PurAH. Asp7Val could not be processed, which may implicate Asp7 in metal coordination. While presence of a heterocycle within the S1 site (Using standard protease nomenclature) of PurAH was essential for activity, the S1' site is more flexible, as demonstrated by partial conversion of BotA mutants Met9Ala and Met9Phe. In all cases where turnover was observed, only the mass for heterocyclized, macrocyclized product could be detected. These data demonstrate that PurAH is promiscuous in the context of single amino-acid changes, while retaining a high fidelity for macrocyclized substrate.

Scheme 2. (a) Proposed mechanism of follower peptide removal by PurAH. It is unclear which residue stabilizes the oxyanion. Zn^{2+}-coordinating residues His94 and His229 have been omitted for clarity (b) Proposed role of PurAH in bottromycin biosynthesis. Removal of the follower peptide (green) by PurAH prevents reopening of the macroamidine (cyan) and thus drives biosynthesis.



It has been demonstrated for amidohydrolases distantly related to PurAH that the aspartate residue involved in metal coordination at the active site (Asp348 in PurAH) and a tyrosine residue in proximity of the active site were involved in catalysis (e.g.[29]). In PurAH, the mutation Asp348Asn resulted in a loss of catalytic activity (Figure S21). These data may implicate Asp348 as the base in catalysis, but we cannot exclude disruption of metal binding. The only tyrosine residue in the immediate vicinity of the active-site Zn^{2+}-ions was Tyr185, and in PurAH the mutant Tyr185Phe showed impaired substrate processing (Figure S21).

We have demonstrated that removal of the follower peptide in bottromycin biosynthesis is catalyzed by PurAH. Since the preceding macroamidine formation is reversible, this enzyme has a profound effect on bottromycin biosynthesis by cooperating with the YcaO enzyme PurCD to ensure efficient macrocyclization. This resolves the apparent disparity between *in vitro*[8-9] and *in vivo*[13] data and places PurAH as the gatekeeper between primary and secondary modification steps (Scheme 2). It also adds to our understanding of the YcaO superfamily: Thioamide-forming YcaO enzymes appear to require a TfuA protein for activity[30], while YcaO enzymes installing heterocycles in linear azolic peptides require an E1-like protein[31-32] and their activity appears in some cases coupled to an FMN-dependent oxidase[33-34]. Despite the cooperation between PurCD and PurAH, we were unable to detect complex formation *in vitro* using various methods (*data not shown*). This raises questions regarding the potential colocalization of RiPP enzymes within the producing organism, which have only been addressed in a very limited number of RiPP systems and require intensive further study. The selectivity of PurAH for macrocyclized precursor peptides coupled with its substrate promiscuity will be important in future efforts to derivatize bottromycins.

ASSOCIATED CONTENT

Supporting Information.
The Supporting Information is available free of charge on the ACS Publications website.
Detailed experimental procedures and supporting information (PDF). The modelled enzyme-substrate complex is available (PDB file).

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Notes

No competing financial interests have been declared.

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